Master's Thesis

A MICROFLUIDIC LEUKOCYTE ROLLING AND ADHESION ASSAY FOR RAPID INFECTION DIAGNOSIS USING A FINGER-PRICK VOLUME OF BLOOD

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Approved by

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Abstract

Sepsis is a severe clinical syndrome triggered by a pathogen invasion into an organism and the resulting subsequent inflammatory response of the body's immune system to it. For long time now, the state of the art method for infection diagnosis was based on blood culture, turnaround time of which (7 days) considerably delayed clinical decision making and prevented timely administration of the antibiotics. We developed a new method for early sepsis diagnosis from a drop of blood, based on upregulated expression of cell adhesion ligands on leukocytes and their resulting differential capture to the biomimetic microfluidic device.

We found that expression of PSGL-1, a primary ligand that enables leukocyte binding to P-selectin and rolling on endothelial bed, is upregulated in response to systemic infection. This finding led us to hypothesize that the higher expression of PSGL-1 on activated leukocytes would result in elevated frequency of leukocyte recruitment and capture on the activated endothelium when perfused with infected blood samples. We developed an inflamed endothelium-mimicking surface by functionalizing the aldehyde-coated glass substrate of the microchannel with a set of the cell adhesion molecules (CAMs) (P-selectin, E-selectin, ICAM-1). We found that when perfused with the infected blood samples, higher percentage of the leukocytes were captured on the biomimetic surface than when perfused with the healthy blood samples. Further validation of our method allowed us to differentiate rats infected with a range of Gram-negative, Gram-positive bacteria and endotoxin from models of sham-infected and healthy rats.

Because the on-chip assay we propose is capable of detecting infection as early as 1-hour post-infection and requires as little as 50 μl of blood for analysis, we believe that this platform could significantly benefit the point-of-care diagnostics of sepsis in a portable device.

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Technical Terms and Abbreviations

PSGL-1: P-selectin ligand-1

ESL-1: E-selectin ligand-1

LFA-1: Lymphocyte functional antigen-1

ICAM-1: Intercellular Adhesion Molecule 1

APTES: (3-Aminopropyl) triethoxysilane

BSA: Bovine serum albumin

PBS: Phosphate-buffered saline

MPO: Myeloperoxidase

PDMS: Polydimethylsiloxane

PCR: Polymerase chain reaction

PCT: Procalcitonin

CPR: Cytochrome P450 Reductase

PFA: Paraformaldehyde

1. Introduction

The successful development of the body's immune response to an infection by a pathogen is possible due to timely recruitment of immune cells to the sites of infection. This multi-step process is regulated by formation of highly specific interactions between the cell adhesion molecules expressed by the endothelium of the infected vessel and the corresponding receptors of the leukocytes[1]. The extravasation cascade is preceded by upregulation of the endothelial expression of cell adhesion molecules, such as P-selectin, E-selectin and ICAM-1, which together orchestrate the series of transient cell-to-cell interactions ultimately leading to migration of the leukocytes from blood to the infected tissue[2]. Constitutive expression of ligands for P-selectin and E-selectin on the leukocytes, named PSGL-1 and ESL-1, respectively, has been reported to be responsible for initiating the instantaneous recruitment of the leukocytes from blood to the endothelium of the vessel underlying an infected tissue[3]. Upon completion of the selectin-dependent rolling, leukocytes firmly adhere to the endothelium via formation of strong bonds between the integrin LFA-1 of the leukocytes and endothelial ICAM-1[4]. Although the components of the immune system that participate in leukocyte recruitment and extravasation were well studied, the development of an *ex vivo* microfluidic system for monitoring the leukocyte-endothelial interactions would further advance the works targeting development of new sepsis diagnosis methods.

The systemic inflammatory response, which follows the septic infection, is severely dysregulated by the host immune system. It is responsible for sever damages in the infected body, such as tissue injury, organ failure and death[5]. The importance of the early infection diagnosis and timely assignment of appropriate treatment is further emphasized by the fact that sepsis-caused mortality grows by 8% for every hour of delay of antibiotic administration[6]. The conventional approach for infection diagnosis is based on blood culture, which on top of being extremely time ineffective (>7 weeks), is known to result in frequent false negative and false positive test results[7]. Several other state-of-the-art methods that rely on utilizing PCR and PCT, CRP assays are limited by high cost, requirement for trained personnel to operate, and most importantly by turnaround times that significantly exceed the "golden hour" requirement[8]. As such, it is estimated that the delay of proper therapeutics for sepsis treatment from the golden hour is responsible for a vast majority of the hospital mortalities and affects the lives of 31.5 million people around the globe[9]. The critical need for a rapid diagnostic tools for testing large populations of patients is particularly severe when we face a new dangerous pathogen, like in the recent COVID-19 outbreak. Timely clinical decision-making in this settings is strongly limited by the relatively low sensitivity of the commonly practiced PCR and CT tests, and their unavailability for the point-of-care testing needs.

We created a microfluidic system that allows rolling and adhesion of leukocytes to the CAMs functionalized surface to mimic the rolling and adhesion to inflamed endothelium for rapid diagnostic purposes. The reason for lack of sepsis diagnosis devices based on leukocyte adhesion mechanism is explained by the

presumption that the leukocyte extravasation is primarily regulated by the upregulation of the cell adhesion molecules on endothelium, not by the regulation of the cell adhesion receptors on leukocyte surface. We report for the first time that PSGL-1, a primary ligand of the P-selectin, is upregulated by the onset of infection in rat infection models, which allows for assessment of the activated state of the leukocytes based on differential frequency of their adhesion to the CAMs functionalized surface.

2. Materials and Methods

2.1 Preparation of rat infection models and blood processing

The experiments with rats were conducted in accordance with the Institution Animal Care and Use Committee (IACUC) of the Ulsan National Institute of Science and Technology, as well as proper anasthesia, analgesia and aseptic conditions. To develop Gram negative and Gram positive bacteremia models, we intraperitoneally injected rats with 1 mL saline containing *E. coli* and MRSA, respectively, both at 10^8 CFU/mL. The cardiac puncture was used to collect the blood samples from *E. coli* infected rats 1, 4 and 12 h post-infection, while for MRSA model, the blood was collected only 4 h post-infection.

To induce the endotoxemia, we injected rats with Saline containing LPS, in the same fashion as in the bacteremia model, and collected blood samples for experiment after 4 hours. The healthy control model of the rat was free of any injections, whereas the sham model of the rats was injected with 1 mL of sterile Saline.

2.2 Lung immunofluorescence analysis and CAMs expression profile

For the immunofluorescence analysis, septic and healthy rats were euthanized and their lungs were isolated for subsequent fixation in paraformaldehyde (PFA). This was followed by standard paraffin-embedding and sectioning procedures for the subsequent immunofluorescence staining with anti-*E. coli* antibody and Hoechst dye.

Once the blood samples were collected from rats and lysed according to instructions of the lysis buffer supplier, the leukocytes from septic and healthy rats underwent immunocytochemical staining to investigate how expression profile of the adhesion ligands respond to infection. After the leukocytes were fixed by incubation in 4% paraformaldehyde for 10 min, the non-specific binding sites on the surface of leukocytes were quenched with BSA in PBS for 1 hour at room temperature. Fixed and blocked cells were stained in PBS containing Hoechst and Alexa-Fluor-488-conjugated anti-PSGL-1 for staining PSGL-1; Hoechst and Alexa-Fluor-555-conjugated anti-GLG1 for staining ESL-1; CellTracker Green and Alexa-Fluor-350-conjugated anti-CD18 for staining LFA-1; Hoechst and FITC-conjugated rat anti-myeloperoxidase for staining neutrophils. Cells were washed in PBS by centrifugation at 300g for 5 min between each two steps of the staining protocol. For the imaging, stained cells were loaded in hemocytometers and their viability was evaluated by presence of Hoechst or CellTracker signal.

2.3 Device fabrication and functionalization

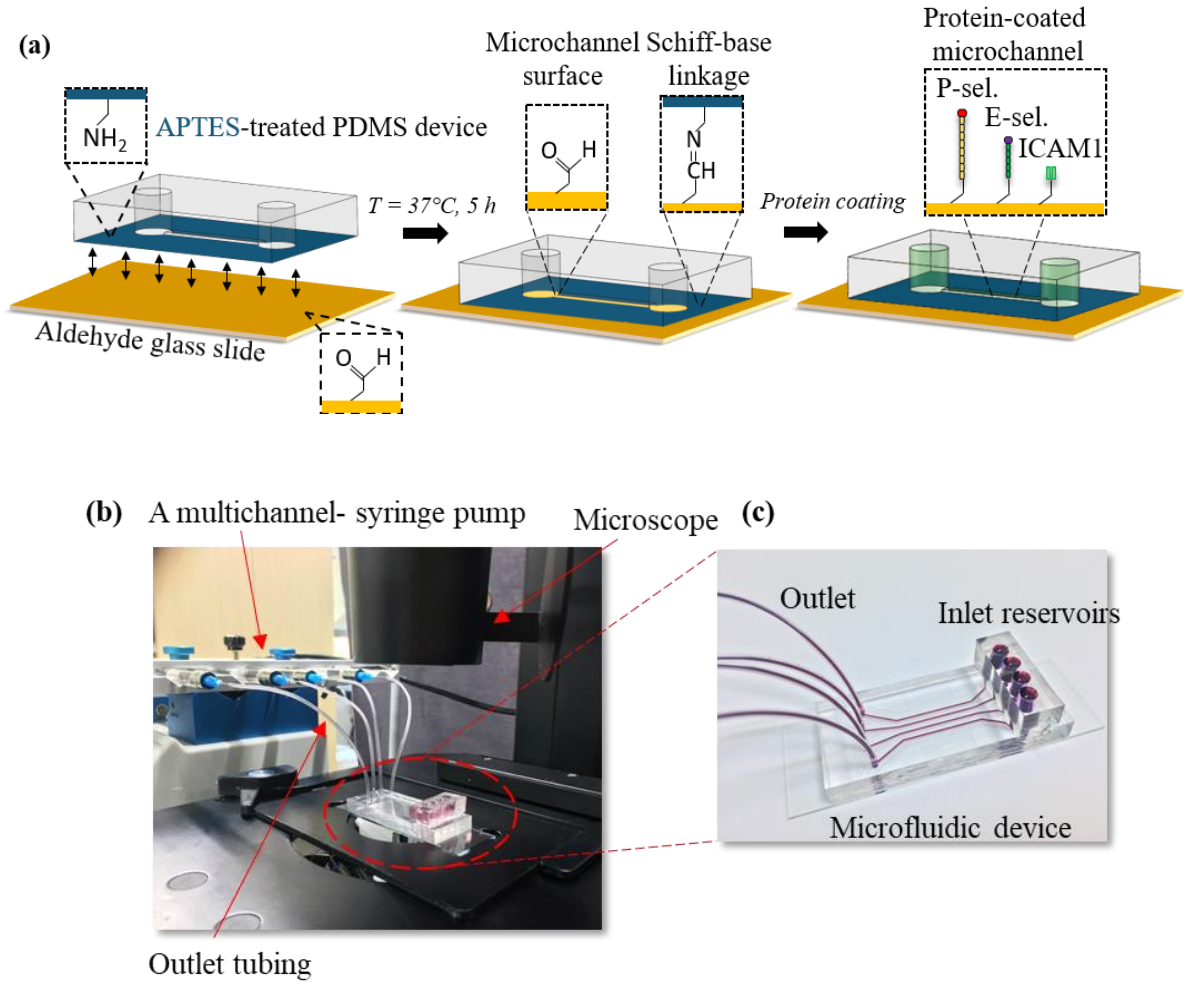

Figure 1. (a) A scheme of the Schiff-base linkage-based chemical bonding of the PDMS device onto glass substrate coated with aldehyde group, and the following attachment of the CAMs onto it. (b) An experimental setting and components. (c) The assembled device for infection detection.

Standard SU-8 photolithography was used to develop the mold for the microfluidic channels. To fabricate the device using the SU-8 mold, poly(dimethylsiloxane) (PDMS) containing the pre-polymer and a curing agent at a weight ratio of 10 : 1 were cast on the mold and cured overnight at 65°C.

Based on a previous work, in which we showed that proteins are effectively immobilized on the aldehyde-functionalized glass slides for immunoassay purposes, we utilized aldehyde-coated glass as a substrate in our microfluidic device.[10] Since plasma treatment was reported to cause irreversible damages to aldehyde-functionalized surfaces, we developed an alternative approach for irreversible chemical bonding of the PDMS device to glass slide. After the surface of the PDMS slab containing the microfluidic pattern is coated with APTES to generate free amino-groups, it is pressed against the aldehyde-coated glass slide, and the assembly is placed at 65° for 5 h to facilitate a Schiff base linkage between the amino groups and

the aldehyde group, respectively (Figure 1a).

Experimental setting (Figure 1b) consists of the microfluidic device for blood analysis, pumps and gastight syringes that control the perfusion of samples into the device via tubing, and the microscope for immediate acquisition of microchannel images for cell quantification. The microfluidic device consists of four identical microfluidic channels with single inlet and outlet (Figure 1c).

2.4 Rolling assay and immunocytochemical staining of captured leukocytes in the chip

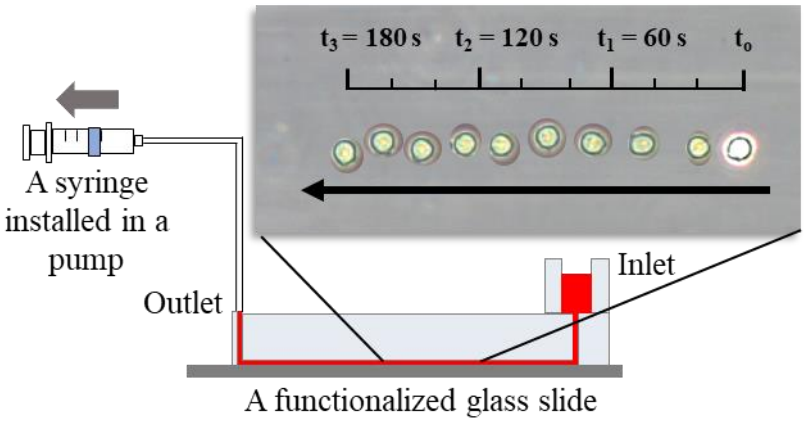

Figure 2. The scheme of the fluidic component that infuses the sample into microfluidic device, thus allowing the rolling of leukocytes over microchannel substrate. The microscopy images of the leukocytes rolling on the channel were superimposed at a time interval of 20 sec.

To differentiate the infection from healthy condition based on the frequency of the leukocyte capture to the CAMs functionalized substrate, we flowed 100 µl of the lysed blood samples from respective experimental groups into the proposed microfluidic device. We mimicked the physiological flow conditions in the microfluidic channel by withdrawing the blood samples at flow rate of 8 µl/min, corresponding to 2 dyn/cm^2 (Figure 2). After 10 min of the withdrawal, the uncaptured leukocytes and red blood cell debris were washed off with 1x PBS and the channels were filled with the CellTracker solution for viability staining of the adhered leukocytes. The frequency of the leukocyte capture was assessed as the percentage of the total leukocyte count that has adhered to the channel substrate upon perfusion, while the total count of the leukocytes that were perfused through the channel was counted by immunostaining of the blood samples with Hoechst and subsequent imaging in the hemocytometer.

2.5 Immunocytochemical staining of the leukocytes

To investigate how the percentage of the neutrophils among the captured leukocytes changes in response to infection, we carried out a immunocytochemical staining of the captured leukocytes with neutrophil-

specific fluorescent antibody immediately after the on-chip perfusion. After filling up the channel at 8 μl/min for 3 min with PFA for fixation of cells, the chip was incubated for 10 min at room temperature. This was followed by consecutive injection of Triton X-100 and BSA into channels to permeabilize the cells and block the non-specific binding sites, respectively. Finally, the cells were co-stained with anti-MPO targeting the neutrophils and Hoechst as viability marker at 4°C for 24 h. Images were obtained using an inverted optical microscope equipped with a digital CCD camera. We identified neutrophils as cells expressing signals for both FITC-MPO and Hoechst.

3. Results and Discussion

3.1 Validation of successful *in vivo* infection in rat model

A successful development of the systemic infection in the animal model was confirmed by immunofluorescence staining of the lung slices isolated from healthy and septic rats with pathogen-specific fluorescent antibody (Figure 3a). We confirmed that the *E. coli*, which was intraperitoneally injected into the abdomen of rat, infiltrated the lungs by 12 hours post-infection, since the staining of lung tissue slices with anti-*E. coli* antibody showed a significantly increased (> 3 fold) fluorescence signal compared to that of the healthy rats (Figure 3b).

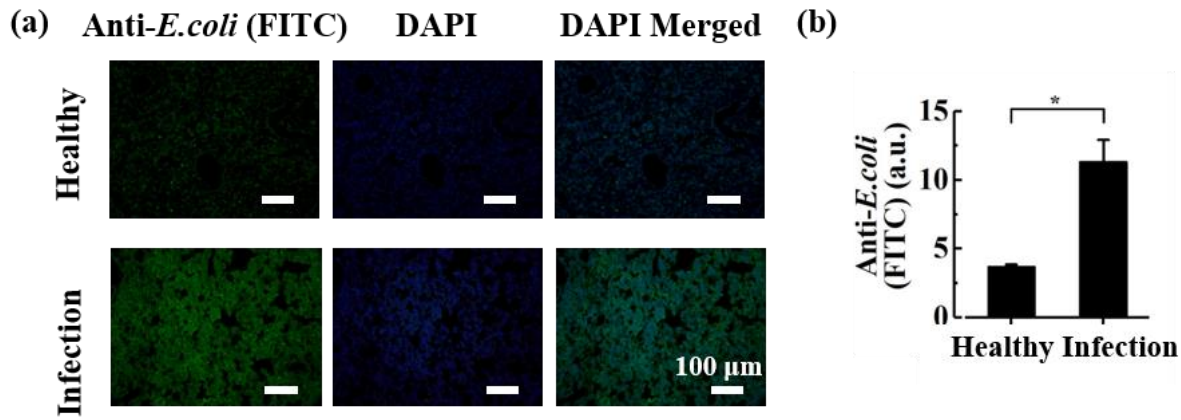

Figure 3. (a) Immunofluorescence microscopy images of the lungs obtained from healthy rats and rats infected with *E. coli* for 12 h, respectively. (b) The increased fluorescence signal emitted by anti-*E. coli* FITC antibody in lung tissue slices isolated from rats infected with *E. coli* for 12 h validates the infiltration of lungs by pathogens in *in vivo* model.

3.2 Characterization of expression profile of CAMs' ligands on leukocytes

We predicted that systemic inflammation triggered by the infection would result in upregulated expression of adhesion ligands on the leukocytes, thus increasing the frequency of their recruitment to the vascular bed near an inflammed tissue via rolling and adhesion on the underlying endothelium. To study how the infection affects the expression levels of the adhesion receptors, we employed a immunocytochemical staining with fluorescence-tagged antibody molecules of the leukocytes extracted from healthy and infected rats (Figure 4a-b). An expression level of PSGL-1, which was evaluated by the detected mean fluorescence intensity (MFI), increased almost by two-fold on leukocytes extracted from *E. coli*-infected rats compared to that of healthy rats (Figure 4c). Although there are numerous articles that argue on up/down modulation of the PSGL-1 expression[11,12], our result clearly shows that PSGL-1 is upregulated as a result of *in vivo* infection. Moreover, this finding is concordant with the previous study of the effect of systemic infection on the expression of cell adhesion receptor in mouse, which reported a significant increase of PSGL-1 expression[13]. Interestingly, in addition to the significantly increased MFI of the stained PSGL-1, the ratio of PSGL-1-expressing leukocytes in blood samples from infected rats were significantly increased compared to that of the healthy rats (Figure 4d). As for ESL-1, although the mean expression level did not exhibit significant differences between the healthy and infected samples (Figure 4e), it was found that significantly higher proportion of the immunostained cells were positively stained for ESL-1 in infected sample compared to healthy sample (Figure 4f). LFA-1, on the other hand, did not show any changes in either the expression level or the percentage of the immunostained cells (Figure 4g-h). Since PSGL-1 and ESL-1 are directly involved and actively facilitate leukocyte rolling and adhesion in the extravasation cascade, we hypothesized that the changes in their expression levels and the elevated percentage of the expressing cells, would significantly affect the frequency of the leukocytes adhesion to the CAMs functionalized substrate.

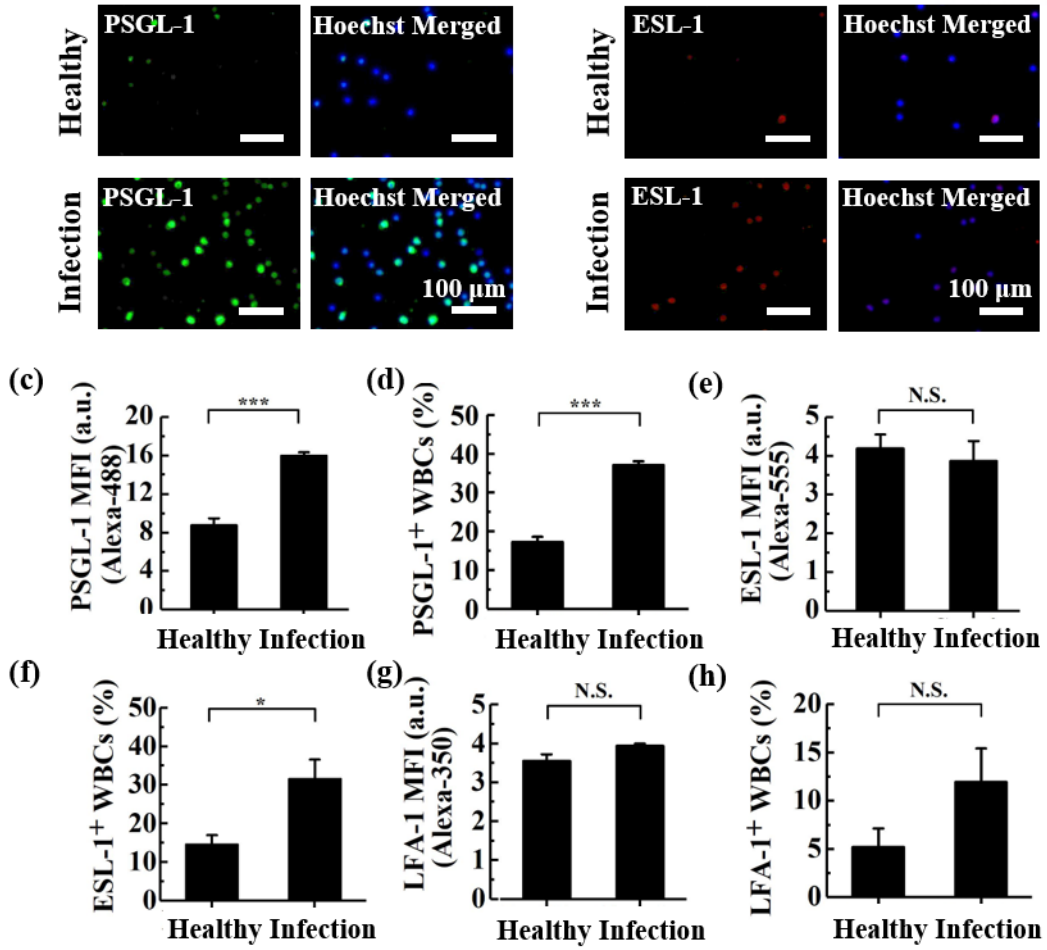

Figure 4. (a and b) Fluorescence microscopy images of the leukocytes from healthy and infected blood samples after their immunochemical staining with fluorescent-tagged anti-PSGL-1 (n = 3; a) and anti-ESL-1 antibodies (n = 3; b). (c to h) Fluorescence signal intensity of the immunostained leukocytes and the ratio of leukocytes expressing PSGL-1 (n = 3; c and d), ESL-1 (n = 3; e and f), LFA-1 (n = 3; g and h) isolated from the septic and healthy rats, respectively, were utilized as quantitative measure of assessing the key leukocyte adhesion receptor responsive to systemic expression.

3.3 Infection-associated neutrophilia in infected rat blood

Although there are numerous reports that PSGL-1 and ESL-1 are expressed by the majority of the leukocytes regardless of their types[14], we predicted that neutrophils, accounting for the primary role they play in the initial inflammatory response, would constitute the higher percentage of the leukocytes expressing PSGL-1 and ESL-1 compared to the healthy rats[15]. Immunocytochemical staining of the leukocytes with anti-myeloperoxidase antibody revealed that the percentage of the neutrophils increases about threefold as the result of infection (Figure 5).

Figure 5. Infection with bacteria was correlated with the significantly elevated ratio of neutrophils, which was revealed by immunocytochemical staining of leukocytes with neutrophil-specific FITC-conjugated myeloperoxidase antibody (MPO).

3.4 Fabrication of functional CAMs-coated microchannels

The microfluidic device that we utilized in this study was designed to mimic the inflammatory endothelium by strong immobilization of the set of P-selectin, E-selectin and ICAM-1, in order to stratify the septic blood from healthy via differential capture of the leukocytes. After PDMS device was fabricated by standard photolithography[16], the bottom surface containing the microfluidic pattern was incubated in APTES for aminosilane functionalization. The PDMS device was bonded to the aldehyde-coated glass slide via formation of Schiff-base linkage between the aminosilane groups and the aldehydes. Since we sought to leverage the functionality of the aldehyde slides in strong immobilization of the cell adhesion molecules, we avoided using destructive oxygen plasma treatment for device bonding. Once the blood samples were lysed and dispensed into inlet reservoirs, leukocytes were withdrawn into the microchannel at physiological shear stress of 2 dyne cm^2[17]. We observed that leukocytes in healthy blood samples moved in stepwise rolling strokes as they were withdrawn over the CAMs-coated surface. The small fraction of rolling leukocytes eventually adhered to the surface via formation of interactions between LFA-1 and ICAM-1 of the leukocyte and the CAMs-coated microchannel, respectively. On the other hand, perfusing the septic blood samples over the CAMs-coated microchannel resulted in significantly higher percentage of leukocytes that adhered to the surface. Thus, we were able to prove the validity of our hypothesis that sepsis can be distinguished from heathy condition based on differential frequency of leukocyte capture to the microchannel coated with CAMs.

Furthermore, we sought out to confirm if the specific combination of three CAMs as P-selectin, E-selectin and ICAM-1 was critical in allowing the differentiation of sepsis (Figure 6a). Functionalization of the microchannel with E-selectin and ICAM-1 showed significant differentiation of the sepsis from healthy condition, but it captured significantly lower percentage of the leukocytes compared to the proposed Condition 1 (Figure 6b). This proved that although PSGL-1 is upregulated on the surface of leukocytes, it

fails to facilitate as effective capture of cells as in Condition 1, due to absence of P-selectin on the surface. Interestingly, the perfusion experiment with the microchannel coated with only ICAM-1 had barely any leukocytes captured to the surface for both experimental groups (Figure 6b), which evidenced the role selectins play in successful transitioning of cells from free flow in channel to strong adhesion.

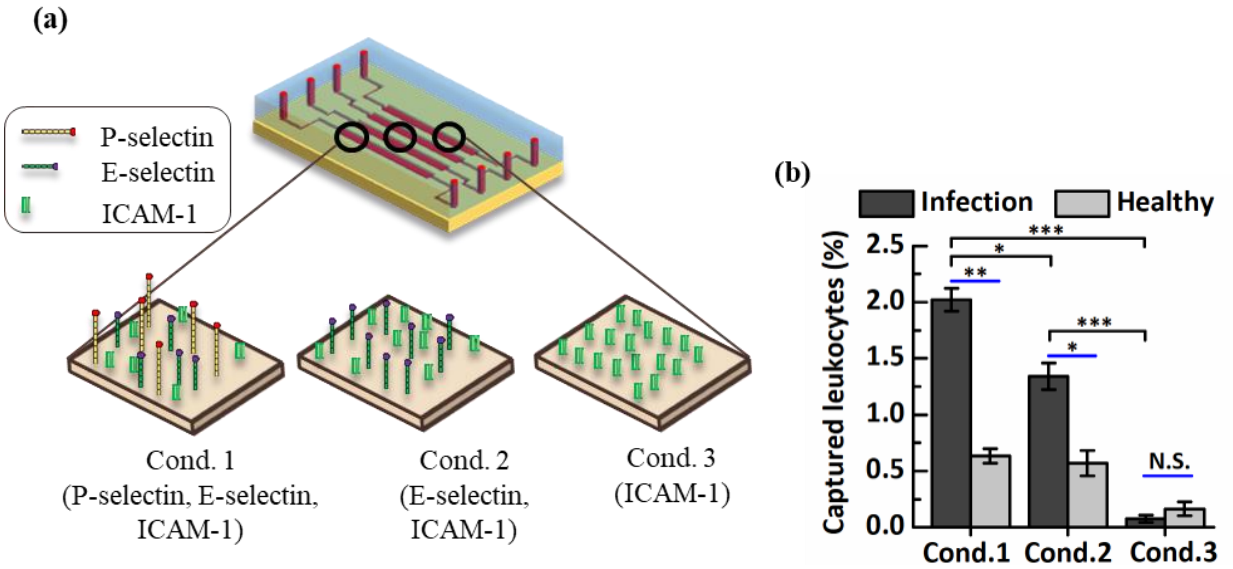

Figure 6. (a) Three combinations of cell adhesion molecules (CAMs) coated surfaces were tested for leukocyte adhesion assay (condition 1: ICAM-1, E-selectin, and P-selectin; condition 2: ICAM-1 and E-selectin; condition 3: ICAM-1). (b) The percentage of leukocytes that were captured to the surface of microchannel coated with different CAMs, from infected and healthy blood samples.

3.5 Neutrophil ratio among captured leukocytes

Based on the findings of PSGL-1 upregulation and elevated percentage of PSGL-1 and ESL-1 expressing cells in the septic blood, as well as accompanying neutrophilia, we hypothesized that perfusion of the infected blood samples over the CAMs coated microfluidic channel would result in higher percentage of the neutrophils among the captured leukocytes. Immunostaining of the leukocytes after their capture on the microchannel with Hoechst and MPO revealed that perfusion of the septic blood samples results in more than half of the captured leukocytes being neutrophils, while constituting only quarter for perfusion of the healthy blood samples (Figure 7).

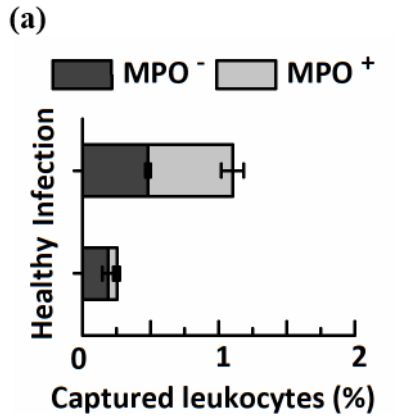

Figure 7. Significantly higher percentage of neutrophils were captured to microchannel from infected blood samples compared to healthy samples.

3.6 Correlation of infection with duration of post-infection time course

The severity of sepsis is correlated with the duration of the infection after the pathogen administration[18], so we sought to investigate how the frequency of leukocyte capture to CAMs would be affected by varying the time between the intraperitoneal injection of *E. coli* and blood collection. As it is often reported that the severity of the sepsis increases with the infection time, we draw blood at 1 h, 4 h, and 12 h after the onset of infection. We found that the differential capture frequency of the leukocytes from blood samples collected 1 h post-infection, significantly stratified sepsis from healthy condition, and further increased at 4 h post-infection (Figure 8). Interestingly, the proportion of the leukocytes captured decreased from 4 h post-infection to 12 h post-infection, which can be explained by a possible down-regulation of the pro-inflammatory processes in septic rats, which has been described previously in related works[19].

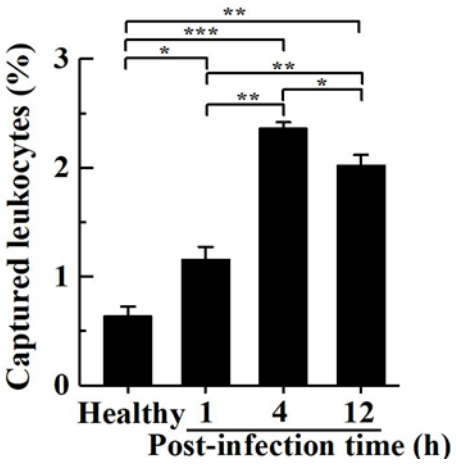

Figure 8. The percentage (%) of leukocytes that were captured on the CAMs-coated microchannel upon perfusion from blood samples of rats infected with *E. coli* for 1 h, 4 h and 12 h.

3.7 Comparison to conventional blood culture technique

To validate the performance of our sepsis detection approach, it was compared to the traditional blood culture method, commonly employed by the hospitals across the world. The results of both the on-chip assay and the blood culture methods tested with blood samples from septic and healthy rats are given in Table 1. Unlike the on-chip approach that can significantly stratify sepsis, the blood culture method gave only "negative" result for blood samples that were obtained 12 h after the onset of infection. The main feature that sets our method apart from the conventional approaches is that it allows sepsis detection within a period of as short as one hour, which allows significantly faster decision-making in the hospital setting compared to approach such as blood culture, PCR and spread plate technique. It also requires only 50 l of blood for analysis, which is considerably lower than the amount commonly withdrawn from patients for the conventional blood culture method[20].

Infection detection methods	**12 h post-infection with *E. coli K12***	**Number of independent trials**
Microfluidic assay	Positive	Sepsis $n = 3$
	Negative	Healthy $n = 3$
BACTEC blood culture	Negative	Sepsis $n = 3$
		Healthy $n = 3$
Spread plate	Negative	Sepsis $n = 3$
		Healthy $n = 3$

Table 1. The utility of the proposed microfluidic assay and clinical blood culture techniques in detecting infection from rat blood samples 12 h post-*E. coli* injection. Positive: Statistically significant differentiation of infected rats from healthy rats was validated ($p < 0.05$); Negative: Statistically significant differentiation was not found.

3.8 Utility of the proposed method in detecting infection by other pathogens

Since sepsis is a complex clinical syndrome that can be caused by a wide range of pathogens, we investigated if our method would be applicable for detecting sepsis induced by pathogens other than *E. coli*. The rats were infected with MRSA and *E. coli*-derived LPS for generating the Gram-positive bacteremia and endotoxemia models. The blood samples were withdrawn 4 h post-infection and used for perfusion over the CAMs-coated microchannel. The on-chip analysis was able to differentiate with statistical significance blood samples of both MRSA and LPS-infected rats from that of the healthy and sham-infected rats (Figure 9).

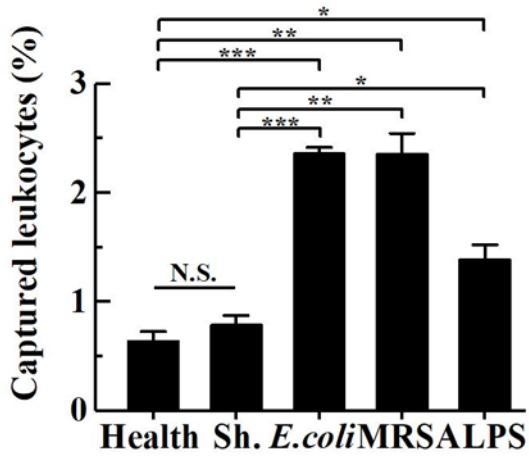

Figure 9. The percentage of the captured leukocytes (%) in the infected models triggered by *E. coli*, MRSA and endotoxin (LPS), which is distinguishable from the control groups (healthy and sham-infected groups).

3.9 Potential applicability for point-of-care diagnostics

We next assessed the applicability of our method with the brightfield microscopy for quantification of cells, not requiring the fluorescent optical setting, as it commonly required for integration of the detection platforms for the operation of point-of-care diagnostics machines (Figure 10a). The automated quantification of the brightfield-imaged cells using the plug-in of the ImageJ software showed no significant differences from the quantification that used the images obtained using fluorescent microscopy (Figure 10b-c).

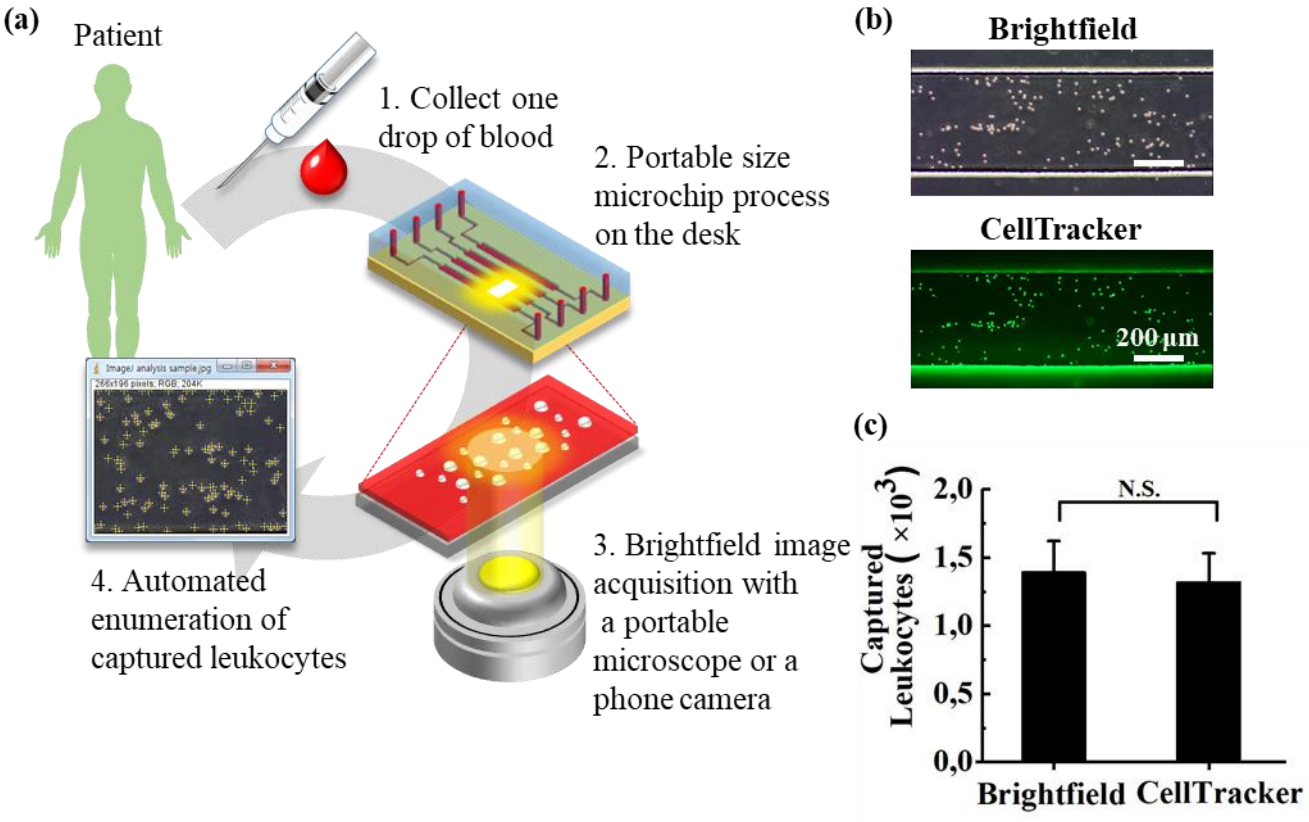

Figure 10. (a) Data acquisition process via a brightfield microscopy, commonly utilized for point-of-care diagnostic purposes. (b) Microchannel segment imaged by brightfield and fluorescent CellTracker™ microscopy methods, respectively. (f) Leukocytes' count extracted via automated quantification of captured cells from brightfield and fluorescent microscopy images

4. Conclusion

One of the initials reactions of the organism during the systemic sepsis is the activation of the endothelium at the sites of infection via expression of cell adhesion molecules, such as P-selectin, E-selectin and ICAM-1. Although the process of the upregulation of these molecules was well investigated in the prior works, the mechanism that modulates the recruitment of the leukocytes from the bloodstream to the infected tissue, as well as the regulation of the expression of receptors complementary to endothelial cell adhesion molecules is left unexplored. We found that the recruitment of the leukocytes for extravasation is regulated not only by the upregulation of the adhesion molecules on the endothelium, but also by the modulation of expression of their respective receptors on the leukocytes. As such, we have found that the population of the leukocytes actively expressing the primary ligands of P-selectin and E-selectin: PSGL-1 and ESL-1 is significantly elevated as a result of infection, respectively. We leveraged these findings to design a novel microfluidic device for infection diagnosis from a drop of blood. Although there were several different works published in the recent years that proposed microfluidics methods for detection of infection based on changes in the expression of cluster of differentiation (CD) [21-22], our method is distinguished by its ability to detect sepsis as early as an hour post-infection, which is considerably faster compared to traditional methods, like blood culture and PCR.

Apart from the on-chip blood analysis, the proposed method requires conducting off-chip procedures, such as RBC lysis in the tube and leukocyte quantification in a hemocytometer. One way to avoid the requirement for these procedures would be an integration of microfluidic module for pre-processing of the blood samples, which would significantly simplify the method and shorten the operation time. We showed that the expensive fluorescence imaging component of the method can be omitted, as we validated that leukocyte quantification is possible with brightfield imaging. This significantly simplifies the process of optimizing the proposed device for development of point-of-care testing system.

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